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(54) Title: PROCESS FOR DETECTING OR QUANTIFYING A BIOLOGICAL REACTION USING SUPERPARAMAGNETIC LABEL

(57) Abstract: A process is described in which superparamagnetic particles are first conjugated or adsorbed to a group of identical biomolecules, such as a group of antibodies, and the conjugates or adsorbates are then reacted with a group of biological binding partner molecules to form a tightly bound, three-dimensional mass of interlinked biomolecules and bound superparamagnetic particles. The mass is exposed to a magnetic field for the shortest period need to induce magnetization of the superparamagnetic particles and the field is then immediately removed. The superparamagnetic particles in the mass exhibit in concert a measurable nonpermanent aggregative magnetization for a period of at least twenty minutes which can be used to quantitate the amount of biological binding partner present by comparison with preestablished standards or to confirm the presence of a biological binding partner in a test sample.

## PATENT APPLICATION

This application is a continuation-in-part of pending U.S. application No. 09/692,463 filed October 20, 2000.

The present invention relates to a new detection system for recognizing and/or monitoring biological, including biochemical, reactions and especially immunochemical reactions. In this system, the introduction of superparamagnetic force effectively facilitates accurate detection of the degree of such a reaction based upon the concentration, or number, of molecules of one reactant that have participated in the reaction. The invention will find ready application in a variety of in vitro uses, including immunoassays, chromatographic molecular separations, nucleic acid probe analyses, pesticide residue analyses, oligonucleotide probes, and other areas in which biological, including biochemical, reactions are now observed or measured or where such observations and measurements plainly could usefully be made even though not yet reported.

## BACKGROUND OF THE INVENTION

Various references describe the use of certain colloidal metallic particles, and specifically of colloidal gold, as identifiers for unusual cells or molecular entities that may be present in biological tissues and as tags for biological, including biochemical, molecules in a variety of immunoassays, nucleic acid probe analyses, chromatographic separations, oligonucleotide probes and myriads of other specific applications where the monitoring of a biological reaction, the identification of one or more disease-causing organisms, the identification of specific moieties that participate in a particular biological reaction or that may disrupt the normal functioning of such a reaction, the identification of moieties that trigger

pathological reactions, and similar information of a biological/biochemical nature is being sought.

Use of colloidal gold to tag these reactions may, with care and the addition of complicated conditions and steps, be conducted in a manner that yields information of a somewhat qualitative nature. Qualitative uses of these colloidal gold tags however, are more easily availed of and, in general, yield more accurate and useful results than attempts to obtain quantitative information with them. To put it another way, colloidal gold in particular offers outstanding advantages as tag material when the goal is to use it to identify specific biological molecules or moieties, but obtaining reliable quantitative information from assays and other reactions where only colloidal gold tags are used is most often an extremely daunting and time-consuming task.

A number of suggestions have been made for employing magnetic beads or particles as labels for biological reactions, on the premise that magnetic field sensors will yield readings enabling determinations of, e.g., molecular size or yield of desired end product. See, e.g. Adelman U.S. Patent 5,656,429 and Adelman, L., J. Assn. for Laboratory Automation 4, No. 3, pp. 32-35 (July 1999).

The Adelman journal article teaches that off-the-shelf magnetic beads exhibit "remnant [sic] magnetization, i.e., are permanently magnetizable" (p. 33) and that their remanent magnetic field is what enables quantitation and/or detection of polynucleotides and other bound targets. In its examples the beads referred to are of an unusually large size in the order of 800 nm in one instance and 4 microns in another. Such large beads cannot effectively be used as markers for the great bulk of biological reactions because their

rheological properties prevent their ready movement through the normally somewhat viscous media in which such reactions occur, and also prevent their ready flow along matrices such as cellulose derivatives, paper, wood, glass etc. upon which such reactions are often performed. The article further teaches that scientists in biomedical and biotechnology laboratories have long recognized a need, when using magnetic beads, for separating excess unbound beads from those that become bound to target whole cells, DNA or proteins -- and that this separation is performed by subjecting the mixture to a fixed magnetic field which attracts the unbound beads (Id.) It is noted that both the permanent magnetizability of these beads and the circumstance that a fixed magnetic field attracts the excess beads strongly suggest the beads were ferromagnetic in character and not superparamagnetic.

Baselt U.S. Patent 5,981,297, proposes using magneto-resistive elements, similar to those used for reading magnetic tapes or disks in, e.g., electronic and computer applications, which are described as measuring approximately 20 by 20  $\mu\text{m}$ . (Col. 6 line 34) to detect many particles per element, (as distinguished from a one particle-per-element embodiment also proposed). This magnetoresistive element is first precoated with an insulator and a binding molecule specific to the target molecule is then covalently bound thereto. The thus prepared element is placed in a flow cell to which liquid sample containing target molecule is added, followed by addition of a suspension of 1-5 nm diameter particles that may be ferromagnetic, ferrimagnetic, superparamagnetic or paramagnetic and have a coating of the binding molecule specific to the target molecule. The specification teaches (Col. 7, lines 1-14) that each magnetoresistive element has the

purpose to count the particles that bind to the target molecule, and that prior to activating the magnetoresistive detection element, a magnetic device, such as an electromagnet is used to remove non-specifically adhering particles. It says this function is "best provided by sending a brief (~10-100 ms) pulse of current generated by a capacitive discharge circuit, through an air core electromagnet coil" (Id., lines 11-14). A magnetic field generator (Col. 7, lines 21-38) then magnetizes the bound beads, each of which creates a magnetic field that changes the resistance of the magnetoresistive element to which it is bound and the resistance is then compared to that of a reference element by a Wheatstone bridge, whereupon the data are digitized and conveyed to a microprocessor which determines the total number of beads on the particular magnetoresistive element, from which target molecule concentration can be calculated.

Various other ways of using magnetic or paramagnetic beads to measure a target substance within a sample have also been described. See, e.g. Rapoport U.S. Patent 5,978,694 involving the use of an electrical conductor to measure changes in magnetic susceptibility of a liquid sample when the latter, having present a paramagnetic, diamagnetic or ferromagnetic labelling material (which may be oxygen, which is characterized as "paramagnetic") that is bound at least in part to a target substance, is subjected to an applied magnetic field.

A German group Kotitz *et al.* have demonstrated that ferromagnetic (including ferrimagnetic nanoparticles can be used to tag various biomolecules). See the Kotitz *et al.* abstract "Superconducting Quantum Interference Device-Based Magnetic Nanoparticle Relaxation Measurement as a Novel Tool for the Binding Specific Detection of Biological

Binding Reactions" J. Appl. Phys. vol. 81, p. 4317 (April 1977) which relates to a conference paper given in November 1996 at the 41st Annual Conference on Magnetism and Magnetic Materials, held at Atlanta, Georgia, the related U.S. Patent 6,027,946 which claims a German priority date of January 27, 1995 and names as inventors the same four individuals named as authors on the abstract. Also closely related to the published abstract and the U.S. patent are a further conference paper from the same group published in IEEE Transactions on Applied Superconductivity vol. 7, no. 2, pt.3, pp. 3678-81 (1997) and first given at the 1996 Applied Superconductivity Conference in Pittsburgh, Pennsylvania in August 1996 and a later article from the group published in J. Magnetism and Magnetic Materials vol. 194, no. 1-3, pp. 62-68 in April 1999. In all of these four cited publications the group worked with ferromagnetic, including ferrimagnetic, nanoparticles rather than with superparamagnetic particles. Three of these publications including the U.S. Patent, relate to the detection of analytes labelled with these ferromagnetic materials using a detection method the authors (inventors) term "magnetorelaxometric" wherein SQUIDS are used to measure time in milliseconds within which the ferromagnetic particles, after exposure to, and withdrawal of, a magnetic field -- both usually done within a magnetically shielded environment -- undergo at least a partial reordering of their magnetic moment.

The IEEE Transactions paper, relates to using SQUIDS to measure remanent sample magnetization in the absence of an external field. More specifically, this paper describes a process wherein monoclonal antibodies to collagen Type III were coupled to dextrane - coated ferromagnetic iron oxide nanoparticles having an average mean diameter of 13 nm. Meanwhile polystyrene tubes were incubated with collagen type III in PBS, whereby this

antigenic material adsorbed onto the tube walls.

The ferromagnetic nanoparticle-labelled monoclonal antibodies in a ferrofluid were added to these prepared tubes and allowed and allowed to incubate for 60 minutes. The tubes were each exposed to a magnetic field for 10 seconds. A measurement of magnetic remanence was then made on the ferrofluid - filled tubes, the measurement being performed in a magnetically shielded environment. The tubes were then each decanted and washed three times with PBS and a magnetic remanence measurement was again made on each. The results showed no change in the remanence signals from those obtained while the tubes were still filled with ferrofluid. From this it was concluded that unbound particles, -- i.e. particles that did not participate in the antibody-antigen reaction -- could be wholly disregarded whether or not they had initially reacted with monoclonal antibody alone to form "blocked particles" and that the measured remanent effect was solely discernible with particle labelled antibodies that bound to the adsorbed antigen on the cell walls. This measured remanent magnetization, moreover, was found to be a linear function of antigen concentration the tube walls.

The present invention involves the discovery that individual superparamagnetic particles of physical size such that they exhibit average mean diameters between 1 and about 100 nm, preferably between 1 and about 60nm, and most preferably from 5 nm to 50 nm are not permanently magnetizable (and hence do not possess the remanent magnetization described in the IEEE publication) but nevertheless do acquire, when closely packed together in an interlocked bio-organic matrix, an impermanent magnetization effect that persists long enough to permit informative and highly useful measurements to be made.

## BRIEF DESCRIPTION OF THE INVENTION

The present invention involves using superparamagnetic particles having a physical size as measured by X-ray diffraction and transmission electron microscopy wherein the average mean diameter is in the range from 1 to about 100 nm, preferably 1 to about 60 nm and most preferably between about 5 nm and 50 nm, as labeling agents for at least one selected or suspected reactant of a biological reaction wherein the reactants, upon interaction among or between them, form a three-dimensional mass of tightly packed, often molecularly cross-linked, bioorganic material and bound superparamagnetic (SPM) particles. In such a mass, the SPM particles are closely juxtaposed to one another in all three dimensions of the mass and the result is that, upon subjecting the mass to the magnetizing effect of a magnetic field (e.g., the field exercised by a magnet of about 10,000 Gauss in strength) for a short period in the order of not more than 30 seconds, preferably 10 seconds or less, the closely juxtaposed particles become magnetized.

It has been experimentally shown that this magnetization gradually decays over a period of at least 20-30 minutes and in some cases, longer, to a point where it disappears. The magnetization described in the preceding sentence is referred to herein as "nonpermanent aggregative magnetization." This non-permanent aggregative magnetization is desirably measured within a set time interval, in minutes, of the withdrawal of the influence of the magnetic field from the mass.

By standardizing this interval to 5 minutes during the work underlying this invention, it was found that a standard curve could be constructed for particles having the same identity characteristics (i.e., particle diameter, surface treatment, and identity of



biomolecule to which it is bound) that permits ready calculation of the number or concentration of target molecules that reacted with these particles and were accordingly present in the test sample.

In these experiments it was also found that stray individual particles of superparamagnetic material having a physical size with the same average mean diameter range and having the same surface treatment, whether initially bound to a biomolecule identical to those that actually reacted with the target molecules of the test sample or wholly unbound, did not retain measurable magnetization upon withdrawal of the magnetic field. Because they did not, it was concluded to be unnecessary to perform any step of physically separating them prior to proceeding with measurement of the nonpermanent aggregative magnetization of the agglomerated interacted mass of superparamagnetic particles and bioorganic molecules. The latter produce measurable magnetic readings, it is theorized, because of their lower sensitivity to thermal effects. The unreacted superparamagnetic labelled antibody or other biomolecule has a smaller physical particle size compared to the superparamagnetic-labelled immunocomplex or other superparamagnetic-labelled reacted biomass. The superparamagnetism of the labelled antibody or other biomolecule and the direction of its magnetization are more susceptible to immediate dissipation as a result of thermal effects and, just as in the superparamagnetic particle alone, it is believed that the direction of magnetization in the superparamagnetic-labelled antibody tends to become random very quickly.

## BRIEF DESCRIPTION OF THE DRAWINGS

The attached drawings are as follows:

Figure 1 is a schematic diagram of a hysteresis curve for typical ferromagnetic material taken from the literature. In the diagram,  $H$  represents the applied magnetic field in oersteds and  $\sigma$  represents the magnetization resulting therefrom. Magnetic saturation is represented by  $\sigma_s$ , while the remanence, or magnetic induction remaining after removal of the magnetic field is represented by  $\sigma_r$ . The reverse magnetic field necessary to bring  $\sigma_r$  to zero is represented by  $H_c$ , the coercive force.

Figure 2 is a superimposed plot of the measured hysteresis of superparamagnetic particles coated with small polyacrylic acid and then reacted with a commercially available antibody called bethyl. Three such measurements were made on identically treated particles of three different iron concentrations as measured on particles in suspension -- i.e. 2.4 mg/ml. (circles on the plot), 1.0 mg/ml. (squares on the plot) and 0.3 mg/ml. (triangles on the plot). In the plot  $M$  represents the applied magnetic field in emu (electromotive units) per cc. (" $\text{cm}^3$ ") while  $H$  is the coercive force in oersteds necessary to impose magnetization (rightward direction from the zero line) or reverse magnetization (leftward from the zero line).

Figure 2A is a similar plot of superimposed hysteresis measurements made using two different instrumental measuring techniques on polymer coated -- (i.e., small polyacrylic acid-coated or chondroitin sulfate A-coated) superparamagnetic  $\text{Fe}_3\text{O}_4$  particles which particles before coating were identical to those used in the experimental work described herein and a hysteresis measurement made with a Vibrating Sample

Magnetometer ("VSM") on superparamagnetic particles of  $\text{Fe}_3\text{O}_4$  conjugated to CPS antibody. In Figure 2A, the polymer-coated superparamagnetic  $\text{Fe}_3\text{O}_4$  particle measurements made using a SQUID instrument are represented by solid black squares; the measurements made on polymer-coated superparamagnetic  $\text{Fe}_3\text{O}_4$  particles are represented by white circles and the measurements of superparamagnetic  $\text{Fe}_3\text{O}_4$  particle-antibody conjugate are represented by solid black triangles.

Figure 3 is a plot of measured magnetization in relative magnetic units vs. antigen concentration in ng/ml of two series of superparamagnetic particle:antigen:immobilized antibody sandwiches as measured at the capture line of an ICT device of the type described, e.g., in U.S. Application Serial No. 07/706,639 of Howard Chandler, now U. S. Patent \_\_\_\_\_, or any of its various continuing patents and applications, all of which are assigned to Smith Kline Diagnostics, Inc. but exclusively licensed to the assignee of this invention, Binx, Inc. for immunological assays over a wide subject matter area. These data were obtained from work described in Example 3.

Figure 4 is a plot of Fe concentration, calculated as  $\text{Fe}_3\text{O}_4$ , of the superparamagnetic particle labels used in Example 3 in  $\mu\text{g/ml}$  against measured CHW antigen concentration in ng/ml. This data was also obtained from work described in Example 3.

Figure 5 is a plot of measured magnetization in relative magnetic units against antigen concentration in pg/ml. It also embodies data from work described in Example 3.

Figure 6 is an X-ray diffraction diagram showing superimposed results of measuring particle size of the superparamagnetic  $\text{Fe}_3\text{O}_4$  particles used in the experimental mental work

herein described (pattern A) the same particles after coating with one of the two polymers identified above (pattern B).

#### DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, superparamagnetic particles which are individually too small to maintain any degree of magnetization after exposure to the action of a magnetic field of the strength of about 10,000 Gauss for a period as short as possible, preferably 10 seconds or less, and not more than 30 seconds, have been shown to acquire measurable non-permanent aggregative magnetization -- i.e., collective magnetization of an aggregated, interacted three dimensional biomass -- when closely incorporated into a tightly packed three-dimensional mass with agglomerated biological material such as, e.g., a mass of labeled superparamagnetic antibody:antigen:immobilized antibody "sandwiches", a clotted mass of labelled blood platelets, a mass of chromatographically separated protein, and the like.

Use of superparamagnetic particles as labels for biological, including biochemical, reactions offers substantial advantages over many of the labels now used, e.g., in various assay systems. For example, superparamagnetic particles, in contrast to ferromagnetic particles, do not display remanent magnetization and have no magnetic properties until subjected to the influence of a magnetic field. They are accordingly virtually unlimitedly shelf-stable in contrast to many of the labelling materials in common use, including colloidal metals, enzymes, chemiluminescent agents, radioactive tracers etc.

Their stability renders them easy to mix with other substances, to suspend freely in liquids and otherwise to work with, so long as they are not exposed to magnetic fields of

sufficient intensity to excite magnetization.

Nonpermanent aggregative magnetization as observed in the context of the present invention is a measurable phenomenon which is a straight line function of the concentration, or number, of target biomolecules in a test sample. However, care must be taken to measure the nonpermanent aggregative magnetization at the same time interval after removal of the magnetic field that causes this magnetization if one is to achieve comparable results in a series of tests — e.g., tests conducted at different concentrations of a target analyte molecule, tests undertaken to construct a standard curve, tests undertaken with the intent to rely on an already constructed standard curve to determine concentration present in a sample, etc.

It is believed that particle size, surface features of the particles, magnetic field strength and time employed in the magnetization step, as well as the mean distance between the magnetized particles trapped in the end product mass of bioorganic material and bound particles, will all play a role in the length of time within which nonpermanent aggregative magnetization persists and the rate at which it decays. It also appears that the decay, at least in systems so far tested, occurs at a rate such that correlation of the number of bound particles with the concentration of a target analyte or other target molecule can be achieved when total magnetization measurements are made not only at the 5 minute interval following the magnetization step that was chosen for the work underlying this invention but at some other uniform interval from that step. Care must be taken, of course, that the measurements are taken at an interval such that measurement of total magnetization yields readings that are in excess of the reading for any background magnetization that may need

to be deducted, depending upon the "platform" or biological matrix that may be present. Furthermore, before selecting a different interval for measurement of total magnetization, one needs to ensure that the rate of decay of non-permanent aggregative magnetization follows a consistent pattern for superparamagnetic particles that have the same treatment history.

As used herein, "superparamagnetic particles" refers to particles that are magnetizable but retain no permanent magnetization when tightly packed together in close association in a mass of inter-reacted bio-organic materials and that, when measured individually after attempted magnetization, exhibit no remanent magnetization. These particles may comprise pure metals such as, Fe, Co, Cd, Ru, Mg, Mn, etc. that are known to be readily magnetizable, iron oxide,  $\text{CoFe}_2\text{O}_4$ ,  $\text{MgFe}_2\text{O}_4$  and oxides of other metals that are known readily to be magnetizable when a mass thereof is subjected to the influence of a magnetic field, such as nickel, cadmium, cobalt, ruthenium, etc. Also usable are, e.g. Fe-Ru and its oxides and other metal combinations, and oxides thereof, that exhibit spinel structure upon examination by X-ray diffraction and Transmission Electron Microscopy. It should be noted that pure metals are superparamagnetic only within a physical size range wherein the average mean diameters are confined with a few nanometers, usually less than 5 nm. Superparamagnetic particles of pure metals are also chemically unstable whereas oxides of corresponding superparamagnetic particles are relatively inert and maintain their superparamagnetism within a broader physical size range.

These superparamagnetic particles are not intrinsically reactive with bio-organic materials and often are desirably coated with a substance that enables them to react with a

binding partner of the target molecule which is to be monitored, assayed for, or otherwise located and quantitated. Various methods of and materials for such coating are known and have been used in the past for coating polymers or glass, including glass beads and solid polymer - comprising inserts or "dipsticks" that have been used in various assay systems. The same coating methods and materials are useful in coating superparamagnetic particles to be used in detecting end products of biological, including biochemical, reactions. Various methods of adsorption are also well known wherein proteins and the like are directly adsorbed on, e.g. iron oxides and the like and they also may be utilized in this invention to improve the reactivity of the particles.

Superparamagnetic particles are distinguished from both ferromagnetic, including (ferrimagnetic), particles, which acquire permanent remanent magnetization upon exposure to an external magnetic field, and also from paramagnetic materials, which have a positive magnetic susceptibility less than 0.001 times that of ferromagnetic materials. The magnetic susceptibility of superparamagnetic materials lies between that of ferromagnetic materials and particles is intermediate that of ferromagnetic and paramagnetic particles. Ideal superparamagnetic systems, at temperatures equal to or below their critical blocking temperature exhibit a slow relaxation time — i.e., they revert from a magnetized state to a non-magnetized state slowly. The particles used in the work underlying this application were of 5-15 nm average mean diameter as measured by X-ray diffraction and Transmission Electron Microscopy and exhibited a blocking temperature slightly above room temperature, i.e., slightly above about 25° C. They were of pure  $\text{Fe}_3\text{O}_4$  having spinel structure, as confirmed by X-ray diffraction and transmitted electron microscopy.

Superparamagnetic materials are known by physicists not to exhibit remanent magnetization. The hysteresis loop of superparamagnetic materials (i.e., the plot obtained by plotting magnetization against magnetic field strength) is curve-like and it typically resembles those shown in Figures 2 and 2A hereof. This is in contrast to the typical hysteresis loop of ferromagnetic materials (Figure 1) and the linear hysteresis plot obtained with paramagnetic materials. Most usually, superparamagnetic particles have a small average mean diameter in the order of less than 50 nm, often 30 nm or less, in physical size as measured by X-ray diffraction and Transmission Electron Microscopy -- although in some systems larger sizes of particles with superparamagnetic properties have been observed. (It is noted parenthetically that both their size and behavior after removal from a magnetic field suggest that the 800 nm and larger particles referred to in the Adelman *et al.* article were ferromagnetic and not superparamagnetic). Still further, it is typical of superparamagnetic particles that the magnetization they may exhibit when subjected to a magnetic field, decays with time until it dissipates altogether. Finally, superparamagnetic particles possess a degree of magnetic ordering - i.e., they have what is called a subdomain structure consisting of clusters of varying sizes containing some atoms with unpaired electrons in the unmagnetized state, but the clusters are small and scattered in comparison to the "domain structure" of ferromagnetic materials which are characterized by larger clusters called domains in which each atom or other structural unit has unpaired electrons that impart a net magnetic moment. In these latter materials, each domain exhibits a directional magnetic effect which is the vector sum of all unpaired electrons present in that domain.



In sum, ferromagnetic materials have strong magnetic ordering, superparamagnetic materials have some magnetic ordering, but much less than ferromagnetic materials. The presence of magnetic ordering in superparamagnetic materials has been confirmed by neutron diffraction measurements. See Chen *et al.*, "Synthesis of Paramagnetic  $\text{MgFe}_2\text{O}_4$  Nanoparticles by Coprecipitation", J. Magnetism and Magnetic Materials, vol. 94, pp. 1-7 (1999). Paramagnetic materials have no magnetic ordering.

For a good technical description of similarities and differences physicists recognize among "ferromagnetic", "superparamagnetic", and "paramagnetic" materials, see Chen *et al.*, "Size-dependent Superparamagnetic Properties of  $\text{Mg}_2\text{FeO}_4$  Spinel Ferrite Nanocrystallites", Appl. Phys. Letters, vol. 73, pp. 3156-8 (1998).

It is possible that the ability to measure non-permanent aggregative magnetization in agglomerated three-dimensional reaction products comprising bio-organic materials in close association with superparamagnetic particles is attributable to the relatively stable macrostructure of these reaction products, which macrostructure holds the incorporated particles in place and prolongs the decay, of the magnetization imparted by exposure to a magnetic field. Applicants, however, have not established this or any other scientific explanation for the reproducible phenomenon observed in the experimental work relating to this invention and hence do not intend to bind themselves to any particular explanation.

The present invention provides enhancements in a virtually unlimited spectrum of in vitro biological reactions including at least immunoassays, DNA probes, oligonucleotide probes, chromatographic molecular separations and other biological reactions where it is

desirable to quantitate the amount of a target molecule present. It is believed that this invention may also be useful in monitoring certain in vivo biological reactions. The detection system of this invention is beneficially employed in immunoassays generally, but especially in immunochromatographic and other "lateral flow" assays and in so-called "flow through" assays -- i.e., those involving vertical flow steps in which the reactants are brought together.

The Midwest Scientific Co. newsletter, Shark Bytes, for October 2000 describes a form of assay now in development at Ohio State University wherein a compact disc ("CD") rotated by a compact disc player is equipped with tiny reservoirs and channels that cause medical samples suspected of containing target analyte to mix with tiny pools of test reagents. Including superparamagnetic particle tagged binding partners for analytes suspected of being present in tiny pools on such test platforms would lead to very useful assays capable of being rapidly performed and rapidly evaluated via the computer anticipated to be included in the CD player of the Ohio State system. This computer could readily be programmed to read non-permanent aggregative magnetization imparted by an external magnetic field in digital form and to correlate this reading to stored information corresponding to a standard curve. As those skilled in immunoassays will recognize, one CD with associated CD player-computer combination could readily be adapted to perform several assays simultaneously on portions of a single test sample by providing, e.g., different antibodies for different target analytes conjugated to superparamagnetic particles placed in different "pool" regions of the CD.

In addition to CD's, other "platform" materials upon which superparamagnetic

particle-tagged biomolecules may be reacted with target biomolecules in a test sample are contemplated to be useful in work performed within the scope of this invention.

Possibilities specifically explored in preliminary work, in addition to what the specific examples below show, are balsa wood and glass. With balsa wood, it was found that even though the material is intrinsically non-magnetic and non-magnetizable its capillarity may lead to readings of non-permanent aggregative magnetization that exhibit a very large standard deviation. It is believed that filling these capillaries with non-magnetizable plastic or with a substance such as bovine serum albumin, other proteins, polyethyleneglycol or other substances well known for blocking capillarity in "dipstick" type immunoassay devices described in the prior art would render balsa wood more acceptable as a platform. Glass was found to avoid the capillarity problem and to be a satisfactory platform material, provided that appropriate background readings are obtained, allowing one to compensate for the fact that most glass slides contain sufficient iron to be magnetizable to a low degree upon exposure to a magnetic field. This makes it necessary to determine the background signal and subtract it from sample readings whenever biological reactions wherein one reactant is labelled with superparamagnetic particles according to this invention are run on glass as a platform.

To measure non-permanent aggregative magnetization, various instruments may be used. In this regard, several different research and development groups are in process of developing relatively low cost measuring instruments which apply knowledge gleaned from high resolution magnetic recording technology and computer disk drive technology. One of these is the Ericomp Maglab 2000, at least one early prototype version of which is

illustrated in the Adelman J. Assn. for Lab. Automation article cited hereinabove. Another is the Quantum Design, Inc. instrument described in U.S. Patent 6,046,585 issued April 4, 2000.

The following specific examples illustrate the substitution of superparamagnetic labelling according to this invention for colloidal gold in an immunochromatographic ("ICT") assay for Canine Heart Worm that is commercially available from Binax, Inc, assignee of this patent application.

#### Example 1 - Selection of Coating Agent for Superparamagnetic Particles

Superparamagnetic particles can be coated with a reagent that has free carboxyl functional groups in order to be capable of covalent coupling to a particular antibody, such as

the antibody Canine Heart Worm ("CHW"). Initial work was accordingly performed to ascertain the coating material of choice for this purpose.

Because rheological properties are important to the successful operation of ICT assays and past experience with colloidal gold labels has shown smaller particles to be rheologically superior, 10 nm diameter  $\text{Fe}_3\text{O}_4$  particles were chosen for this work. Their size was confirmed by X-ray diffraction and by Transmission Electron Microscopy.

Two polymeric coating materials were tested on separate lots of superparamagnetic particles using the coating method described in U.S. Patent 5,547,682. One lot of these particles was coated with Chondroitin Sulfate A ("CSA") and the other with small polyacrylic acid ("sPAA"). The coated particles in suspension, in each instance had a

diameter of 40 to 60 nm, as determined by dynamic light scattering. Both the CSA - coated and the sPAA coated superparamagnetic particles were further tested and it was shown thereby that CSA - coated particles were superior in stability and ability to bind to antibodies. The hysteresis loop as measured by Vibrating Sample Magnetometer ("VSM") tests for three sets of particles having varying iron concentrations, all of which were sPAA coated and had the commercially available antibody bethyl bonded thereto is shown in Figure 2. These particles in uncoated form are identical to those which were the starting materials for Example 3. Figure 2 shows the typical hysteresis curve shape of superparamagnetic materials and also confirms that these particles had no remanent magnetization. Figure 2A shows hysteresis measurements made with coated particles of superparamagnetic 10nm  $\text{Fe}_3\text{O}_4$  with a SQUID instrument (curve with black squares) and by VSM (curve with white circles). Also, shown on Figure 2A is a plot of hysteresis measurements made by VSM of the same coated particles to which a carboxy polysaccharide antibody was conjugated. All three again exhibit the typical shape of superparamagnetic material hysteresis behavior and confirm the particles lack of remanent magnetization.

#### Example 2 - Preparation of Superparamagnetic Particle - Labelled Antibodies

CSA - coated superparamagnetic particles prepared as in Example 1 were covalently coupled to anti-Canine Heart Worm ("CHW") antibody identical to the anti-CHW antibody used in the manufacture of the commercially available Binax ICT test for CHW antigen and were then suspended in phosphate-buffered saline solution containing 10 mg/ml of bovine serum albumin pending their use as in Example 3.

Example 3 - Performance of ICT Assay for CHW Using Superparamagnetic Labels

ICT flow path test strips of nitrocellulose were treated in the same manner as those used in the Binax commercially available ICT assay for CHW. These strips were incorporated into dipstick-type devices by the lamination of absorbent pad components overlapping opposite ends of the flow path test strip. A series of solutions containing CHW antigen were prepared with antigen concentrations ranging from 100 pg/ml to 200 ng/ml. 190 $\mu$ l of each of these antigen solutions were dispensed into separate wells of a new polystyrene 96 well microtiter plate. To these samples were added 5 $\mu$ l of the CSA-coated superparamagnetic particle-labelled anti-CHW antibodies of example 2. Labeled antibody/antigen solution mixtures were incubated for 15 minutes at room temperature. The sample receiving end of a dipstick device was added to each labeled antibody/antigen solution mixture immediately following this incubation, causing the mixture to flow into the capture zone. Immobilized unlabelled rabbit polyclonal anti-CHW antibodies bound to the strip in the capture zone thereupon reacted with antigen:superparamagnetic labelled antibody conjugates to form immobilized antibody:antigen:superparamagnetic labelled antibody "sandwiches" along the capture line. The ICT strips were removed from the ICT devices after 15 minutes, and exposed to a magnetic field of 10,000 Gauss for 10 seconds each. After 5 minutes from the removal of the magnetic field, each strip was placed in an Ericomp Maglab 2000 instrument with the capture line in the field of view of the detector and its non-permanent aggregative magnetization was read.

Each antigen solution was tested in duplicate in the ICT test as described. The readings of non-permanent aggregative magnetization for both series of sample having known antigen concentrations above 1 ng/ml have been graphed in Figure 3 against antigen concentration. The Fe content of the immune complexes at the capture lines of ICT devices used in the duplicate series of tests was determined by a chemical calorimetric procedure using a commercial ferrizine test from Sigma Chemical Co. It was found that the calorimetric chemical test results and the magnetization readings correlated well, as shown in Figure 4, a plot of iron concentration calculated as  $\text{Fe}_3\text{O}_4$ , in  $\mu\text{g/ml}$ , against antigen concentration in ng/ml.

In the ICT tests as performed in this example, any labelled antibody initially deposited at the flow path threshold that fails to react with antigen in the sample flows past the capture zone and into another pad positioned upstream from that zone. These unreacted paramagnetic particle-labelled antibodies were subjected to the effect of a magnetic field of 10,000 Gauss for 10 seconds, set aside for 5 minutes and then placed in the sensor area of the Ericomp Maglab 2000 instrument and found to exhibit no measurable magnetization.

From the results of the foregoing examples, it was determined that the relationship between magnetic reading in relative magnetic units ("RMU") and concentration of antigen (the target analyte) is an essentially linear function in the range of 1 ng antigen/ml to 150 ng antigen/ml. See Figure 3, a plot of relative magnetic units against antigen concentration in ng per ml for each of the two series of CHW assays performed. The instrument noise, however, caused large standard deviations in the readings of samples having concentrations

of antigen below 1 ng/ml. This is illustrated in Figure 5, a plot of measured values in relative magnetic units against antigen concentration in pg per ml. The fact that ICT strips having 200 pg/ml of added antigen had non-permanent aggregative magnetization that could be read when that antigen was incorporated in labelled antibody:antigen:immobilized antibody sandwiches collected in a mass, subjected to 10,000 Gauss of magnetic field for 10 seconds, and then set aside for five minutes is an indication nonetheless that the sensitivity of the test is significantly enhanced by substituting superparamagnetic labels for colloidal gold labels.

With an improved instrument having reduced noise, it is clear that the physical sensitivity of superparamagnetic labelling as described approaches 0.1 ng of Fe calculated as  $\text{Fe}_3\text{O}_4$  or about  $10^{-18}$  per mole, while the broad dynamic sensitivity range will fall between about 1 and  $10^6$  relative units and has potentially high tolerance to interference from various biological matrices that may present.

While the invention has been exemplified in the context of a well known immunodiagnostic system specific to the antigen of the causative agent for the canine disease Dirofilaria immitis, the vast range of applications in which it will produce greatly improved results or will enable precise quantitative measurement of observed phenomena previously deemed to be difficult to impossible to measure will be readily apparent to those ordinarily skilled in immunochemistry and/or biology. It is accordingly intended that the scope of this invention be limited only to the extent of the scope of the appended claims.



## WE CLAIM:

1. A process for detecting a biological reaction which comprises:
  - (a) conjugating or adsorbing to each of a group of superparamagnetic particles identical biomolecules which are members of a biological binding pair,
  - (b) contacting the product of step (a) with a sample selected from among liquids and solids, containing or suspected of containing molecules which comprise the biological binding partner of the biomolecules conjugated to or adsorbed on the superparamagnetic particles,
  - (c) permitting the superparamagnetic particle: biomolecule conjugates or superparamagnetic particle: biomolecule adsorbates from step (a) to react with any biological binding partner molecules present in the aforementioned sample to form a complex, tightly bound, three-dimensional mass comprising interlinked biomolecules and bound superparamagnetic particles;
  - (d) exposing the said mass to a magnetic field for the shortest period necessary to induce magnetization of the superparamagnetic particles in said mass and then immediately removing the magnetic field, whereupon the superparamagnetic particles in said mass exhibit in concert measurable nonpermanent aggregative magnetization which persists for a period of at least 20 minutes following exposure to the magnetic field, and either
  - (e) confirming the presence of such magnetization with a suitable instrument if only a qualitative result is desired, or

(f) measuring the intensity of the magnetic signal of the said nonpermanent aggregative magnetization before it dissipates and correlating it to the quantitative concentration, or number, of one of the biomolecules of step (a) or step (b) that participated in forming the mass referred to in step (c).

2. A process according to Claim 1 in which the superparamagnetic particles comprise  $\text{Fe}_3\text{O}_4$  particles having an average mean diameter as measured by X-ray diffraction and Transmission Electron Microscopy of 1 nm to about 100 nm.

3. A process according to Claim 2 in which each superparamagnetic particle is conjugated to an antibody, the sample in step (b) is a liquid sample which contains the antigen that is the specific binding partner of said antibody, and a quantitative result is obtained by performing step (f).

4. A process according to Claim 3 in which the period of exposure to a magnetic field in step (d) is 5-10 seconds and the average mean diameter of the superparamagnetic particles as determined by X-ray diffraction and Transmission Electron Microscopy is in the range from 5 nm to 60 nm.

5. A process according to Claim (4) which is an immunoassay in which the antigen content of the sample is quantified in step (f).

6. A process according to Claim 5 which is conducted in lateral flow format.

7. A process according to Claim 6 which is conducted in the format of an immunochromatographic assay.

8. A process according to Claim 5 which is conducted in a vertical flow or flow-through format.
9. A process according to Claim 1 in which the superparamagnetic particles comprise those selected from among superparamagnetic particles of a single magnetizable metal or superparamagnetic particles of two combined magnetizable metals or superparamagnetic particles of oxides of either a single magnetizable metal or two combined magnetizable metals.
10. A process according to Claim 9 in which each superparamagnetic particle is conjugated to an antibody, the sample in step (b) is a liquid sample which contains the antigen that is the specific binding partner of said antibody, and a quantitative result is obtained by performing step (f).
11. A process according to Claim 10 in which the period of exposure to a magnetic field in step (d) is 5-10 seconds and the average mean diameter of the superparamagnetic particles as determined by X-ray diffraction and Transmission Electron Microscopy is in the range from 5 nm to 50 nm.
12. A process according to Claim 11 which is an immunoassay in which the antigen content of the sample is quantified in step (f).
13. A process according to Claim 12 which is conducted in lateral flow format.
14. A process according to Claim 13 which is conducted in the format of an immunochromatographic assay.
15. A process according to Claim 12 which is conducted in a vertical flow or flow-through format.

16. A process according to Claim 9 in which the superparamagnetic particles comprise superparamagnetic particles of an oxide two combined magnetizable metals, which particles exhibit a spinel structure as determined by X-ray diffraction analysis and Transmission Electron Microscopy.
17. A process according to Claim 16 in which each superparamagnetic particle is conjugated to an antibody, the sample in step (b) is a liquid sample which contains the antigen that is the specific binding partner of said antibody, and a quantitative result is obtained by performing step (f).
18. A process according to Claim 17 in which the period of exposure to a magnetic field in step (d) is 5-10 seconds and the average mean diameter of the superparamagnetic particles as determined by X-ray diffraction and Transmission Electron Microscopy is in the range from 5 nm to 50 nm.
19. A process according to Claim 18 which is an immunoassay in which the antigen content of the sample is quantified in step (f).
20. A process according to Claim 19 which is conducted in lateral flow format.
21. A process according to Claim 20 which is conducted in the format of an immunochromatographic assay.
22. A process according to Claim 19 which is conducted in a vertical flow or flow through format.
23. A process according to Claim 2 in which identical biomolecules are adsorbed to superparamagnetic particles in step (a).
24. A process according to Claim 23 in which the period of exposure to a magnetic field

in step (d) is 5-10 seconds and the average mean diameter of the superparamagnetic particles as determined by X-ray diffraction and Transmission Electron Microscopy is in the range of 5 nm to 50 nm.

25. A process according to Claim 24 which is an immunoassay.
26. A process according to Claim 25 which is conducted in lateral flow format.
27. A process according to Claim 26 which is conducted in the format of an immunochromatographic assay.
28. A process according to Claim 26 which is conducted in a vertical flow or flow-through format.
29. A process according to Claim 23 in which the superparamagnetic particles comprise those selected from among superparamagnetic particles or superparamagnetic particles of two combined magnetizable metals or superparamagnetic particles of oxides of either a single magnetizable metal or two combine magnetizable metals.
30. A process according to Claim 23 in which the superparamagnetic particles comprise superparamagnetic particles of an oxide of two combined magnetizable metals, which particles exhibit a spinel structure as determined by X-ray diffraction analysis and Transmission Electron Microscopy.
31. A process according to Claim 1 wherein step f is performed instead of step (e).
32. A process according to Claim 31 which is repeatedly performed on a series of samples each containing different concentrations of a given biological binding partner, as referred to in step (b) of Claim 1, wherein the measurement in step (f) of the intensity of the magnetic signal from the nonpermanent aggregative magnetization of the mass referred

to in step (d) of Claim 1 is performed uniformly for each sample at the same time interval from the time of removal from the magnetic field of exposure of the mass referred to in each of steps (c) and (d) of Claim 1.

33. A process according to Claim 31 wherein, once a correlation has been established between the concentration, or number, of the molecules of given biological binding partner, as referred to in step (b) of Claim 1, and the intensity of the magnetic signal of the nonpermanent aggregative magnetization of the mass containing it referred to in steps (c) and (d) of Claim 1 by measuring said signal in step (f) at a uniform time interval for a series of samples has been obtained as recited in Claim 32, the same uniform time interval in step (f) is adhered to whenever the process of Claim 31 is performed upon any sample containing an unknown concentration, or number, of molecules of the same biological binding partner.

34. A process according to Claim 1 wherein the average mean diameter of the superparamagnetic particles as determined by X-ray diffraction and Transmission Electron Microscopy is in the range of 1 nm to 60 nm.

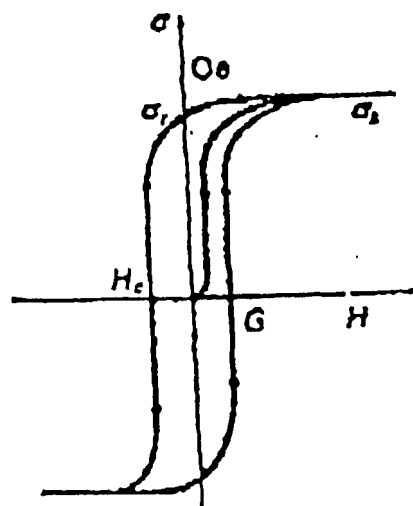


Figure 1

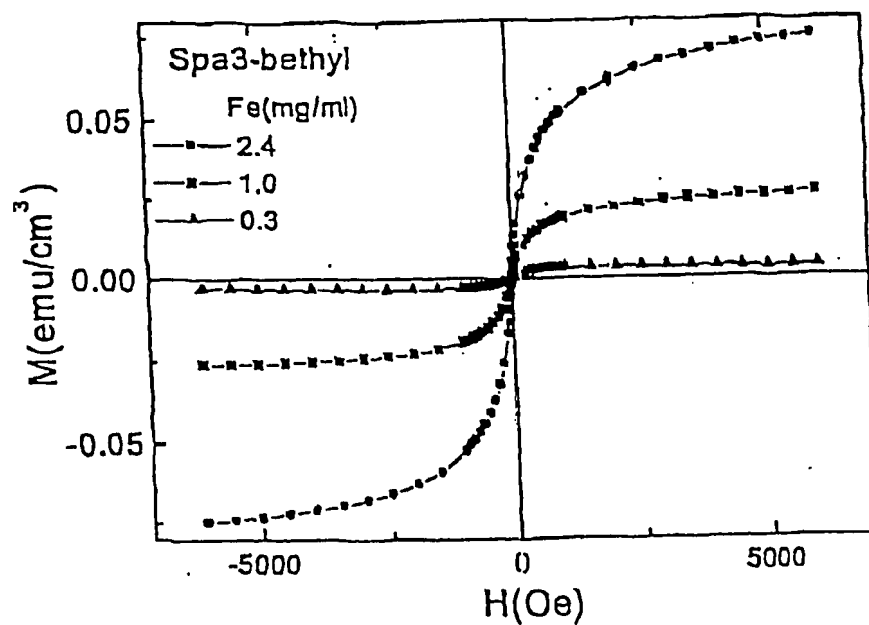


Figure 2

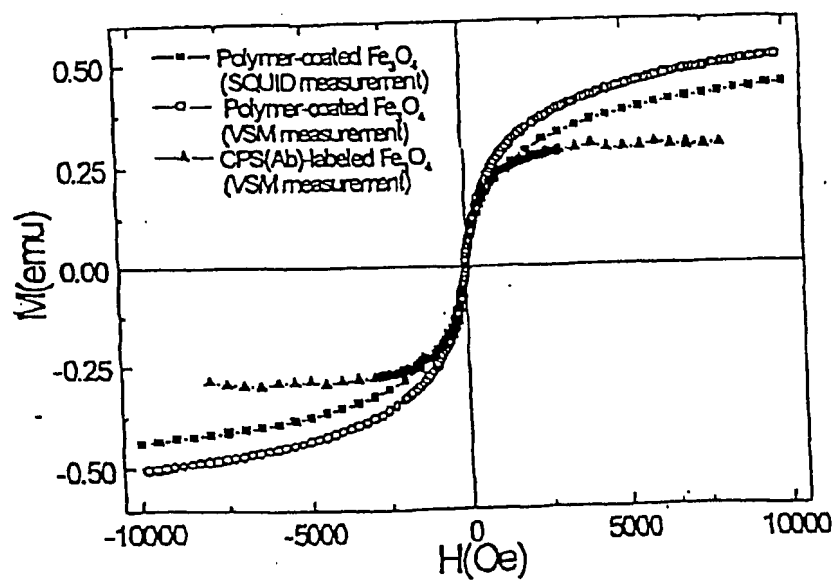


Figure 2A



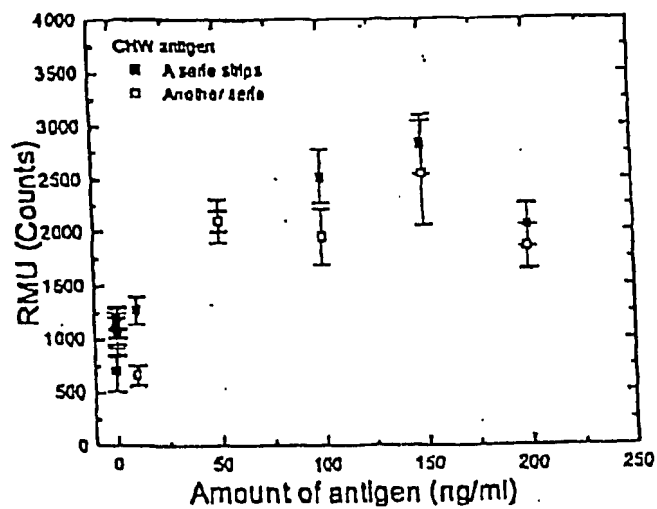


Figure 3

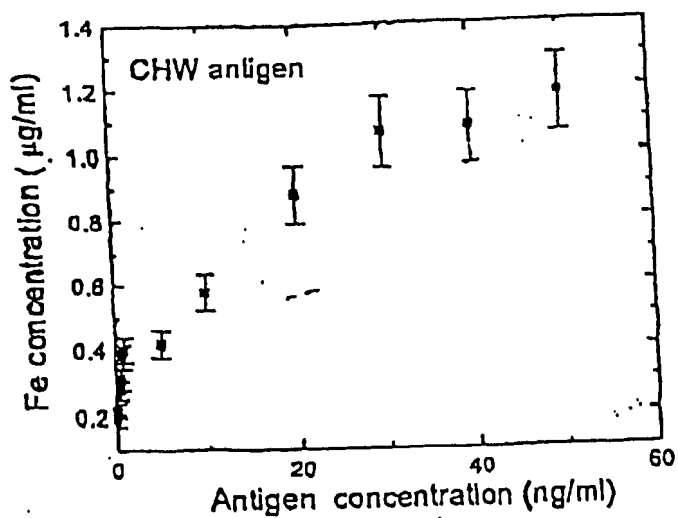


Figure 4

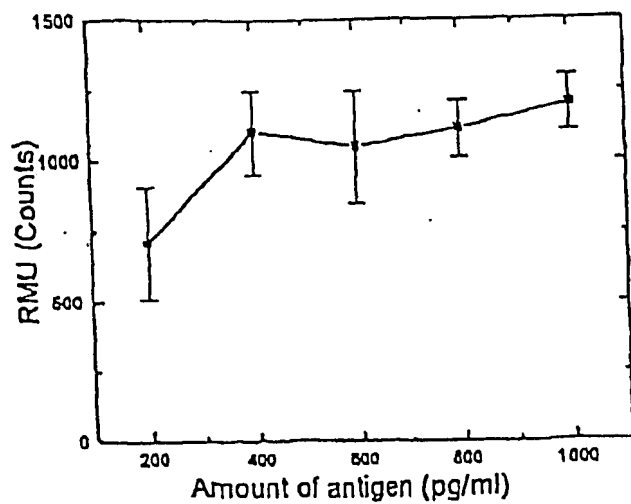


Figure 5

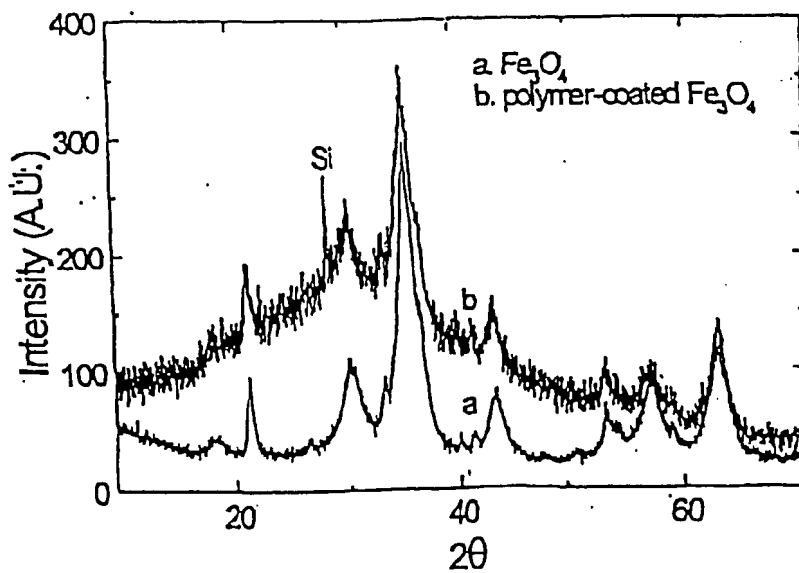


Figure 6